

Heat-shock protein 70 from plant biofactories of recombinant antigens activate multiepitope-targeted immune responses

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Summary

Although a physiological role of heat-shock proteins (HSP) in antigen presentation and immune response activation has not been directly demonstrated, their use as vaccine components is under clinical trial. We have previously demonstrated that the structure of plant-derived HSP70 (pHSP70) can be superimposed to the mammalian homologue and similarly to the mammalian counterpart, pHSP70–polypeptide complexes can activate the immune system. It is here shown that pHSP70 purified from plant tissues transiently expressing the influenza virus nucleoprotein are able to induce both the activation of major histocompatibility complex class I-restricted polyclonal T-cell responses and antibody production in mice of different haplotypes without the need of adjuvant co-delivery. These results indicate that pHSP70 derived from plants producing recombinant antigens may be used to formulate multiepitope vaccines.

Introduction

The main role of HSP, HSP70 in particular, is to assist protein folding and unfolding (Hartl and Hayer-Hartl, 2009) but they have been implicated also in the stimulation of innate and adaptive immune responses (Basu and Matsutake, 2004; Calderwood *et al.*, 2007). This function is tightly related to the noncovalent association of polypeptides derived from protein catabolism to the C-terminal substrate-binding domain (SBD), as the specific immune responses are directed against bound polypeptides (Srivastava, 2002). Polypeptide binding depends on ATP-induced conformational changes (Goloubinoff and De Los Rios, 2007). Even if a physiological role of HSP in the generation of immune responses is still a matter of debate, HSP-based cancer vaccines are currently evaluated in clinical trials (Srivastava *et al.*, 2009). These personalized vaccines consist of extracts prepared from autologous tumour cells containing HSP–polypeptide complexes. Because the polypeptides chaperoned by HSP are expected to be the ‘fingerprint’ of proteins expressed within a cell/tissue at a given time, in this particular case, they are expected to be in part derived by unique tumour-specific antigens (Li *et al.*, 2002).

Because HSP are the most conserved proteins among phyla (Rutherford, 2003), we recently wanted to verify whether plant HSP70 (pHSP70) resemble structurally and functionally their mammalian homologue. Published data demonstrated that the structure of pHSP70 can be superimposed to mammalian HSP70 (mHSP70) and provided the first proof that they are able to activate in mice immune responses by inducing the production of antibodies specific to polypeptides bound to their SBD (Buriani *et al.*, 2011).

The aim of this study was to extend the knowledge on the immunological properties of pHSP70 by evaluating their efficacy in activating cell-mediated immune responses. The data herein

presented indicate that pHSP70 purified from *Nicotiana benthamiana* leaves transiently expressing the nucleoprotein (NP) of influenza A virus are able to induce the activation of major histocompatibility complex (MHC) class I-restricted T lymphocytes. In C57BL/6J mice, the response was demonstrated to be polyclonal and directed against both immunodominant and subdominant NP epitopes. In confirmation of this evidence, the same pHSP70 preparation was demonstrated to be able to activate MHC class I-restricted T lymphocytes also in Balb/c mice. The evaluation of antibody titres in mice sera confirmed that purified pHSP70 are effective also in priming humoral immune responses specific to the plant-expressed recombinant antigen. Remarkably, the responses were achieved without adjuvant co-delivery even after endotoxin removal.

Results

NP expression in plants and HSP70–polypeptide complexes purification from transiently transformed plant tissues

The expression of the NP of influenza virus in *N. benthamiana* leaves was obtained by agroinfiltration. This transient plant transformation method is able to induce the overexpression of both the heterologous protein and the endogenous pHSP70 (Buriani *et al.*, 2011). Plants were infiltrated only with *Agrobacterium tumefaciens* GV3101 (pSOUP) cells carrying the binary vector pGR106-NP or together with *A. tumefaciens* LBA4404 cells carrying the binary vector p35S:p19 encoding the silencing suppressor protein P19 of tomato bushy stunt virus (TBSV). As control, plants were infiltrated with buffer. The aim of these experiments was to define at which time interval and in which conditions (with or without the silencing inhibitor) the expression levels of pHSP70 and NP were maximal. Crude extracts of leaves normalized for total soluble proteins (TSP) content and

sampled at different time intervals from agroinfiltration were analysed by enzyme-linked immunosorbent assay (ELISA) using NP- or HSP70-specific antibodies. The results of these experiments reproducibly demonstrated that both pHSP70 and NP were maximally expressed 7 days after agroinfiltration with both pGR106-NP and p35S:p19 (Figure 1a). These conditions were thus used to extract pHSP70 from tissues transiently expressing NP (NP-pHSP70) to perform the immunological experiments. To immunize a control group of mice, pHSP70 were extracted from wild-type (not agroinfiltrated) *N. benthamiana* leaves (wt-pHSP70).

pHSP70 from crude extracts were purified through ADP chromatography to maintain association with naturally chaperoned polypeptides. Purity and identity were verified through silver-stained SDS-PAGE and Western blot analysis, respectively (Figure 1b). The absence of free NP protein in the final pHSP70 preparation was further confirmed by ELISA (data not shown). Yield was approximately of 23 µg pHSP70/g agroinfiltrated and 14 µg pHSP70/g wild-type fresh leaves. An aliquot of the purified pHSP70-NP was unloaded of peptides through treatment with ATP (un-pHSP70). This peptide-unloaded pHSP70 preparation was used as further control in some of the immunization experiments.

pHSP70 extracted from plant tissues transiently expressing NP activate NP-specific MHC class I-restricted T lymphocytes

To verify the ability of pHSP70 extracted from plant tissues transiently expressing NP to activate NP-specific MHC class I-restricted lymphocytes, C57BL/6J mice were immunized subcutaneously (s.c.) three times (day 0, 7 and 14) with 25 µg wt-pHSP70, un-pHSP70 or NP-pHSP70 in a final volume of 50 µL without adjuvant. An additional group of mice was immunized two times (day 7 and 21) with 50 µg of the synthetic peptide ASNENMETM emulsified in incomplete Freund's adjuvant (IFA) and administered in a final volume of 50 µL. The immunization schedules are schematically represented in Figure 2. At day 28, all the animals were sacrificed, and single-cell suspensions prepared from spleens to set up interferon-γ (IFN-γ) enzyme-linked immunospot (ELISPOT) and *ex vivo* measure the number of T cells responding to the stimulation with different NP-derived nonapeptides known to induce the activation of MHC class I-restricted T cells: (i) ASNENMETM (A; NP_{366–374}), immunodominant epitope in C57BL/6J mice (H-2D^b) (Townsend *et al.*, 1986), (ii) GERQNATEI (G; NP_{17–25}), RLYQNSLTI (R; NP_{55–63}) and YRRVNGKWM (Y; NP_{97–105}), subdominant epitopes in C57BL/6J mice (H-2D^b) (Oukka *et al.*, 1996), (iii) TYQRTRALV (T; NP_{147–155}), immunodominant in Balb/c mice (H-2K^d) (Bodmer *et al.*, 1988). The cells were stimulated with single peptides or with different peptide combinations (G+R+Y; A+G+R+Y; T+G+R+Y; and A+G+R+T+Y). Figure 3 depicts the results obtained in a representative experiment. The first evidence was that the NP-pHSP70 preparation was able to induce a polyclonal response to NP-derived synthetic peptides. This property was not shared by the other pHSP70 preparations (wt-pHSP70 and un-pHSP70). The peptide that reproducibly induced by itself the activation of the highest number of cells in the ELISPOT assay was the immunodominant peptide A. When pools of peptides were used for the *ex vivo* stimulation, significantly higher responses were obtained when the peptide A was included in the peptide mix. The highest response was reproducibly obtained by stimulating the cells with all peptides

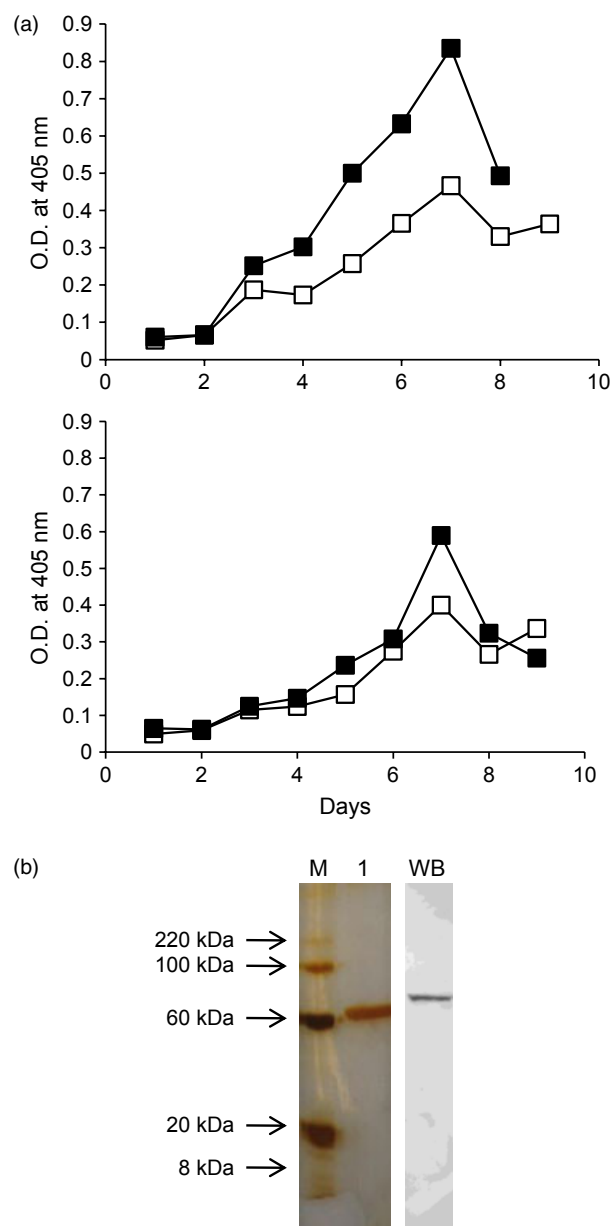


Figure 1 Kinetic of NP and pHSP70 expression in plant tissues and evaluation of the purity and identity of a representative pHSP70-NP preparation. (a) Evaluation through ELISA of the expression levels of NP (upper diagram) and pHSP70 (lower diagram) in *Nicotiana benthamiana* leaf protein extracts (normalized for total soluble proteins content) at different time intervals from infiltration with *Agrobacterium tumefaciens* GV3101 (pSOUP) cells carrying the binary vector pGR106-NP alone (open squares) or together with LBA4404 carrying the binary vector p35S:p19 (solid squares). The results refer to a representative experiment. x-axis: days after infiltration. y-axis: the optical density (OD) values at 405 nm in triplicate wells (after background subtraction) $\pm$ standard deviation. (b) Silver staining of 12.5% SDS-PAGE loaded with a 20-µL aliquot of pHSP70-NP eluted from the ADP-agarose column (left panel, lane 1) or immunoblotting (right panel, lane WB) of an aliquot (150 ng) of the final pooled and concentrated preparation of pHSP70-NP using a monoclonal anti-HSP70 antibody for detection. M: molecular weight marker. Arrows: the molecular weight of marker bands.

(A+G+R+T+Y). This response was significantly higher if compared to that obtained by stimulating cells only with the peptide A. The differences among the responses to the single

subdominant peptides were not as much reproducible as those described above.

The same NP-pHSP70 preparation activates MHC class I-restricted lymphocytes in mice of different haplotypes

The ability of NP-pHSP70 to activate T cells specific to different NP-derived peptides was verified by immunizing with the same preparation and schedule also Balb/c mice. In these experiments, control groups were immunized with the synthetic peptide TYQRTRALV instead of ASNENMETM. At day 28, all the animals were sacrificed, and single-cell suspensions prepared from spleens to set up IFN- γ ELISPOT and *ex vivo* measure the number of MHC class I-restricted T cells responding to the stimulation with single NP-derived nonapeptides (A, G, R, T or Y) or with different peptide combinations (G+R+Y; A+G+R+Y; T+G+R+Y; and A+G+R+T+Y). Figure 4 depicts the results obtained in a representative experiment. Overall, these results

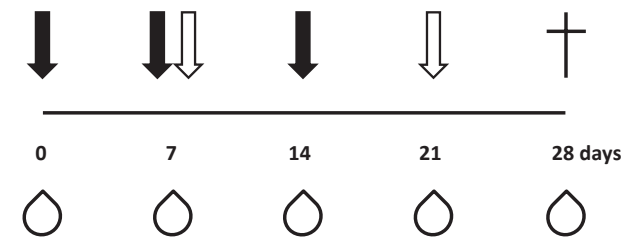


Figure 2 Schematic representation of the immunization and bleeding schedule. Solid arrows: immunization schedule of mice receiving wt-pHSP70, un-pHSP70 or NP-pHSP70. White arrows: immunization schedule of mice receiving synthetic peptides. Drops: bleeding times for serum collection. Cross: sacrifice.

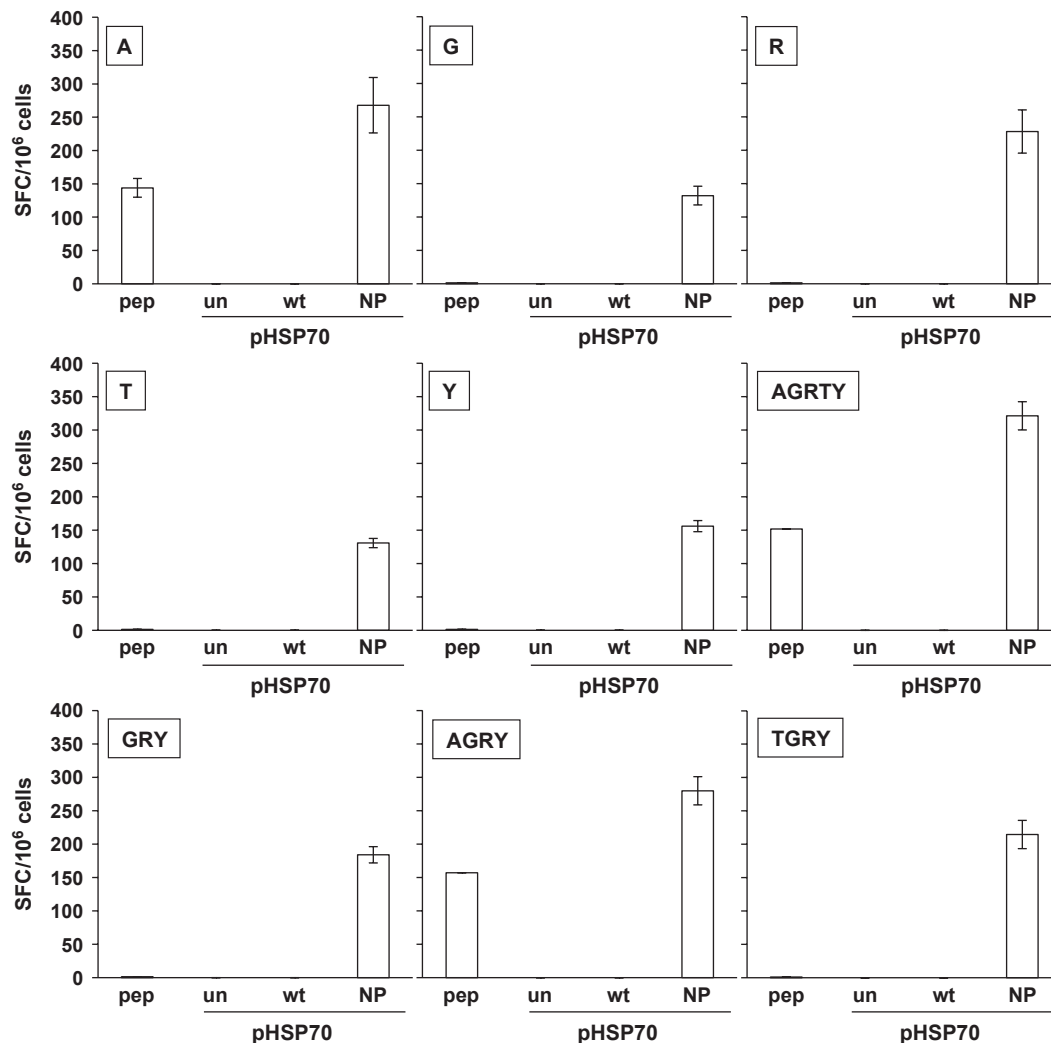


Figure 3 Evaluation by *ex vivo* IFN- γ ELISPOT assay of the activation of T cells specific to NP-derived peptides induced in C57BL/6J mice by immunization with different pHSP70 preparations. Each panel reports the response in terms of spot-forming cells (SFC) of the same splenocyte preparations to different synthetic peptides, singularly (A: ASNENMETM; G: GERQNATEI; R: RLYQNSLTI; Y: YRRVNGKWM; and T: TYQRTRALV) or in combination. pep: mice immunized with the peptide ASNENMETM emulsified in incomplete Freund's adjuvant (three mice/group). un: mice immunized with a pHSP70 preparation *in vitro* unloaded of peptides. wt: mice immunized with pHSP70 purified from not agroinfiltrated leaves (four mice/group). NP: mice immunized with pHSP70 purified from leaves transiently expressing NP (four mice/group). Each value represents the mean of triplicate wells after subtraction of SFC counted in the respective negative control (unstimulated cells) $\pm$ standard deviation.

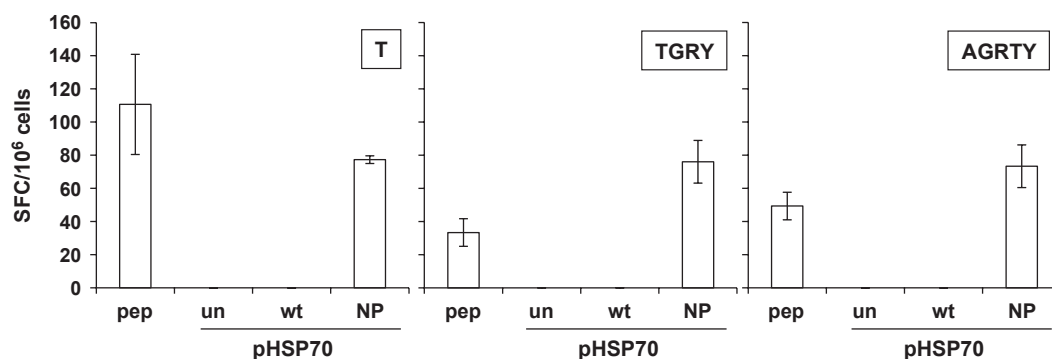


Figure 4 Evaluation by *ex vivo* IFN- γ ELISPOT assay of the activation of T cells specific to NP-derived peptides induced in Balb/c mice by immunization with different pHSP70 preparations. Each panel reports the response in terms of SFC of the same splenocyte preparations to a single peptide or to peptides combination (A: ASNENMETM; G: GERQNATEI; R: RLYQNSLTI; Y: YRRVNGKWM; and T: TYQRTRALV). pep: mice immunized with the peptide TYQRTRALV emulsified in incomplete Freund's adjuvant. un: mice immunized with a pHSP70 preparation *in vitro* unloaded of peptides. wt: mice immunized with pHSP70 purified from not agroinfiltrated leaves. NP: mice immunized with pHSP70 purified from leaves transiently expressing NP. Each value represents the mean of triplicate wells after subtraction of the SFC counted in the respective negative control (unstimulated cells) $\pm$ standard deviation. The response to A, G, R and Y peptides singularly or in combination (G+R+Y; A+G+R+Y) is not reported because they were not different from negative controls.

confirmed that only the NP-pHSP70 preparation was able to induce the NP-specific response and not the others (wt-pHSP70 and un-pHSP70). In this case, the cells responded to the immunodominant peptide T only. No responses were obtained to A, G, R and Y peptides, singularly or in combination (G+R+Y; A+G+R+Y) (data not shown). The number of spot-forming cells (SFC) obtained by stimulating the splenocytes with T+G+R+Y or

with A+G+R+T+Y was comparable to that obtained by stimulating with T only.

NP-pHSP70 immunological properties persists also after endotoxin removal

To rule out a possible role of bacterial endotoxin contamination in the activation of the observed immune responses, this was

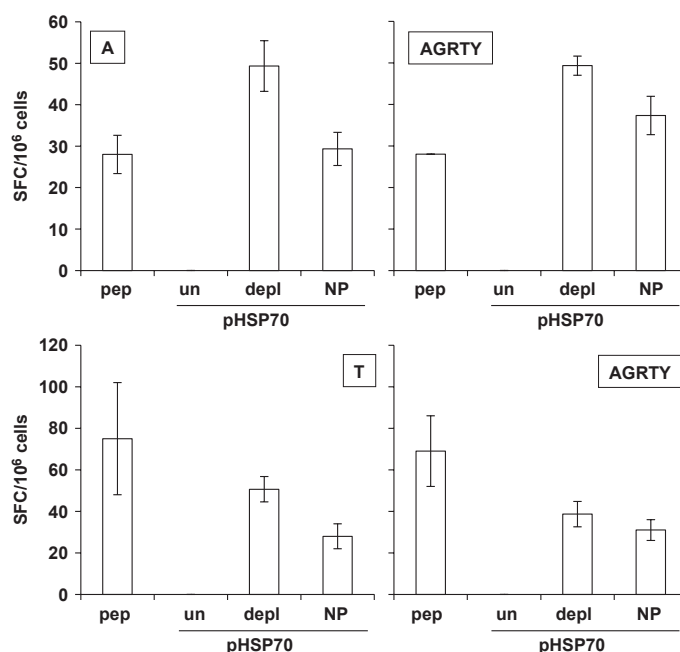


Figure 5 Evaluation by *ex vivo* IFN- γ ELISPOT assay of the activation of T cells specific to NP-derived peptides induced in C57BL/6J and Balb/c mice by immunization with NP-pHSP70 preparations, depleted or not of endotoxin. Upper diagrams: C57BL/6J mice splenocytes response to stimulation with the synthetic peptide ASNENMETM (A; left panel) alone or in combination with the peptides GERQNATEI (G), RLYQNSLTI (R), YRRVNGKWM (Y) and TYQRTRALV (T) (right panel). Lower diagrams: Balb/c mice splenocytes response to stimulation with the synthetic peptide TYQRTRALV (T; left panel) alone or in combination with the peptides ASNENMETM (A), GERQNATEI (G), RLYQNSLTI (R) and YRRVNGKWM (Y) (right panel). pep: mice immunized with the peptides A (C57BL/6J mice; upper panels) or T (Balb/c mice; lower panels) emulsified in incomplete Freund's adjuvant. un: mice immunized with a pHSP70 preparation *in vitro* unloaded of peptides. depl: mice immunized with endotoxin-depleted NP-pHSP70. NP: mice immunized with NP-pHSP70. Each value represents the mean of triplicate wells after subtraction of the SFC counted in the respective negative control (unstimulated cells) $\pm$ standard deviation.

removed from a purified pHSP70 sample through a chromatography-based method. The classical Limulus amoebocyte lysate (LAL)-based gel clot assay indicated that the sample initially contained about 15.4 endotoxin units (EU; 1 EU = 100 pg)/mL, while after column passage, the contamination was lower than 1 EU/mL. Mice (C57BL/6J and Balb/c) were immunized s.c. with 25 µg of NP-pHSP70 depleted (depl-pHSP70) or not of endotoxin, following the schedule previously described. Controls were immunized with 25 µg un-pHSP70 or with 50 µg of synthetic peptides ASNENMETM or TYQRTRALV emulsified in IFA. The response of both C57BL/6J and Balb/c splenocytes to the synthetic peptides in terms of SFC in the IFN- γ ELISPOT assay demonstrated that endotoxin removal did not perturb the immune properties of NP-pHSP70 rather it induces an increase in the response mainly to the immunodominant peptides (Figure 5).

pHSP70 extracted from plant tissues transiently expressing NP activate antibody responses specific to NP

Prior and at different time intervals from immunization, blood samples were individually collected from C57BL/6J and Balb/c mice to quantify antibodies specific to NP or to pHSP70 (see Figure 2 for a schematic representation of bleeding times). Both responses increased as a function of the number of immunizations. ELISA results of sera collected before sacrifice demonstrated that all pHSP70 preparations (un-pHSP70, wt-pHSP70 and NP-pHSP70) were able to induce in mice pHSP70-specific antibody responses, while antibody responses

specific to NP were detectable only in the sera of mice immunized with NP-pHSP70 (Figure 6a). Similar results were obtained also for the sera of mice immunized with endotoxin-depleted NP-pHSP70 preparation (Figure 6b). The absence in the sera of the mice immunized with un-pHSP70 of antibodies specific to NP was a further demonstration of pHSP70 preparation purity.

Because the pHSP70 preparations were expected to carry a complex set of polypeptides, derived not only from NP but also from plant proteins, ELISA was set up to verify whether antibody responses were induced also against these proteins. In these experiments, wells were coated with soluble protein extracts from wild-type leaves, depleted of pHSP70. The results confirmed the presence in the sera of mice immunized with wt-pHSP70 and NP-pHSP70 of antibodies specific to plant proteins, although at titres lower than those specific to HSP or NP (data not shown). These antibodies were absent in control mice immunized with un-pHSP70.

Discussion

To improve the efficiency of vaccination against particular pathogens, such as influenza viruses, there is a palpable need to identify new targets and delivery strategies able to activate broad immune responses (Hedestam *et al.*, 2008). It has been suggested that vaccines against these pathogens beside inducing the production of neutralizing antibodies should activate also immune responses against conserved internal antigens as these responses could be cross-protective in the case of the

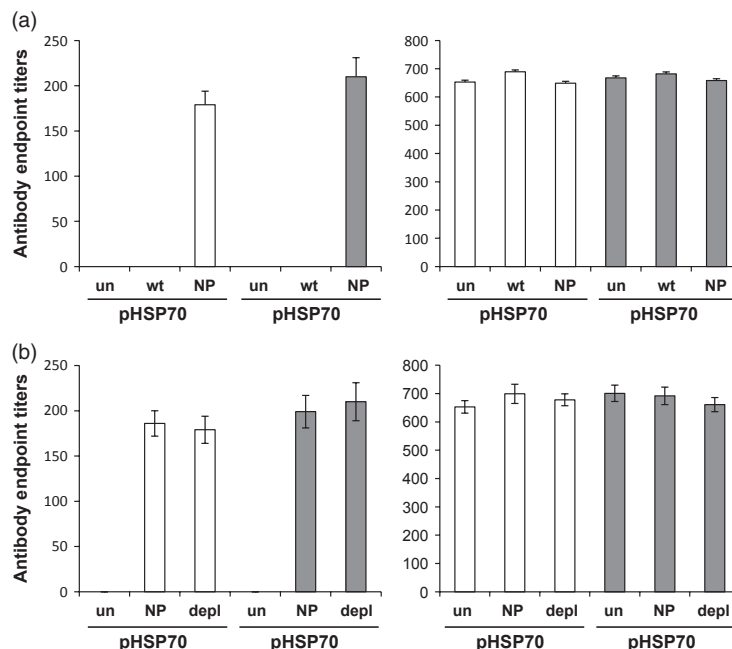


Figure 6 Titration of antibodies specific to NP or to pHSP70 in the sera of mice of different haplotype immunized with pHSP70 preparations and depleted or not of endotoxins. (a) Anti-NP and anti-pHSP70 antibody titres in the sera of mice immunized with different pHSP70 preparations. un: mice immunized with a pHSP70 preparation *in vitro* unloaded of peptides. wt: mice immunized with pHSP70 purified from not agroinfiltrated leaves. NP: mice immunized with pHSP70 purified from leaves transiently expressing NP. Left panel: NP-specific IgG titres. Right panel: pHSP70-specific IgG titres. White bars: C57BL/6J mice. Grey bars: Balb/c mice. (b) Effect of endotoxin depletion on anti-NP and anti-pHSP70 antibody titres in mice sera. un: mice immunized with a pHSP70 preparation *in vitro* unloaded of peptides. NP: mice immunized with pHSP70 purified from leaves transiently expressing NP. depl: mice immunized with endotoxin-depleted NP-pHSP70. Left panel: NP-specific IgG titres. Right panel: pHSP70-specific IgG titres. White bars: C57BL/6J mice. Grey bars: Balb/c mice. Titres are expressed as arithmetic mean of individual mice titres obtained in a representative experiment $\pm$ standard deviation. The differences among each group and controls are significant for $P < 0.05$.

outbreak of viral variants against whom pre-existing humoral immunity is not (or poorly) protective (Doherty and Kelso, 2008). The unequivocal major role played by CD8+ T lymphocytes in protection from infection has been demonstrated by experimental and epidemiological data (Rimmelzwaan *et al.*, 2007).

The identification of novel antigen delivery strategies is another major task especially if among the aims of vaccination, there is the activation of cytotoxic T lymphocytes (CTL). Indeed, these responses can be obtained only if antigens are vehiculated into the cytosol of presenting cells (Kovacsics-Bankowski and Rock, 1995).

A further perceived need concerns production technologies that must be rapid, inexpensive and safe (Rimmelzwaan and Osterhaus, 2001; Osterholm, 2005).

Mammalian HSP-peptide complexes have been demonstrated able to induce the activation of both antibody and CTL responses (Singh-Jasuja *et al.*, 2001) and successfully used for the formulation of autologous cancer vaccines, avoiding the need to identify target antigens (Srivastava *et al.*, 2009). We have recently demonstrated that the structure of pHSP70 is very similar to mHSP70. Moreover, when pHSP70 are extracted through ADP chromatography (thus 'loaded' with peptides) from leaf tissues expressing a heterologous 'reporter' protein, they are able to induce antibody responses specific to this protein in mice (Buriani *et al.*, 2011). The present study was directed primarily to examine whether pHSP70-peptides complexes were also able to induce the activation of CD8+ T lymphocytes. Other fundamental issues to be solved were to verify whether the activated MHC class I-restricted T-cell responses were directed against different epitopes of the same antigen and the same pHSP70 preparation could be able to activate the responses in mice of different haplotypes, in summary to verify whether the preparation could work as a multiepitope vaccine.

The heterologous protein overexpressed in plant leaves was the NP of influenza virus. This antigen was selected to take advantage of literature data available about fine mapping of MHC class I-restricted epitopes in different strains of mice. The expression in *N. benthamiana* leaves of NP was achieved by agroinfiltration. This transient plant transformation method was chosen as an elective method to yield high level of heterologous proteins in plants in few days (Sparkes *et al.*, 2006) and as the most efficient at eliciting the maximum of pHSP70 expression (Buriani *et al.*, 2011). pHSP70 were purified from wild-type plant tissues and from plant tissues expressing NP, through ADP chromatography to avoid the release of polypeptides chaperoned within plant cells. The activation of CD8+ T cells in mice after immunization with these pHSP70 preparations was evaluated *ex vivo* by IFN- γ ELISPOT assays, stimulating the splenocytes with NP-derived synthetic peptides. These experiments clearly showed that NP-pHSP70 were able to induce the activation of CD8+ cells specific to NP in mice. This property was not shared by wt-pHSP70 carrying only polypeptides derived from endogenous plant cell proteins nor by un-HSP70 derived from NP-expressing plant tissues but 'unloaded' of polypeptides by specific ATP treatment *in vitro*. In C57BL/6J mice, it was also possible to demonstrate that the NP-specific response was polyclonal and directed against both immunodominant and subdominant epitopes. The response to the immunodominant epitope A was reproducibly higher than that to subdominants. This could depend on dif-

ferences in the efficiency of HSP-peptide interaction (Javid *et al.*, 2007) and/or in peptide affinity for MHC class I molecules (Oukka *et al.*, 1996). The variability among the responses to the subdominant epitopes was not as much reproducible. This could depend on variability in peptide composition of the pHSP70 preparations used to perform the experiments. The higher response was reproducibly obtained when stimulating the splenocytes with the complete pool of peptides (immunodominant + subdominants).

The polyclonality of the response was confirmed by experiments demonstrating that the same pHSP70 preparation used to immunize C57BL/6J mice was effective in inducing the activation of CD8+ T cells also in Balb/c mice. In both strains, NP-HSP70 maintained their efficacy of inducing CD8+ T-cell activation even after endotoxins removal being also able to induce the activation of antibody responses. The reason why endotoxin depletion induces an increase in the number of SFC mainly in response to the immunodominant peptides has not been determined. Further experiments will be necessary to clarify this issue.

The complexity of the preparation in terms of peptide composition was further tested by experiments aimed to determine whether the purified pHSP70 were able to activate antibody responses specific to plant proteins. The results of these experiments, indicating the presence of these antibodies in the sera of the immunized mice, render mass spectrometry-based catalogue of pHSP70-bound polypeptides one of the primary aim of future experiments also to have indications on the variability in polypeptide composition among different pHSP70 batches. Precious information on the possible side effects deriving from the use of such antigenically complex HSP-based vaccine preparations will derive also from ongoing clinical trials of personalized HSP-based cancer vaccines prepared from autologous tumour cells (<http://www.agenusbio.com/>). Indeed, also in that case, the possibility cannot be excluded that HSP carrying polypeptides derived from nontumour proteins could induce the activation of undesired immune responses against self-antigens (autoimmunity).

The results shown herein extend the knowledge on pHSP70 immune properties in general and in particular highlight the properties of pHSP70-polypeptide complexes derived from plant tissues expressing recombinant antigens. As a whole, the proposed immunization strategy induces both antibody production and CD8+ T-cell activation. The responses are polyclonal and, intriguingly, do not need adjuvant co-delivery. The use of plants as biofactories for the production of recombinant antigens, also of influenza virus proteins has been described (D'Aoust *et al.*, 2008; Shoji *et al.*, 2008; Spitsin *et al.*, 2009). From a general point of view, plant-based production strategies are flexible, safe and rapid. The major drawbacks of this approach concern the purification of the product of interest as the efficiency of this step can vary case by case (Desai *et al.*, 2010). The suggested plant-based approach for antigen production displays all the positive values of plant-based expression systems with the additional clue that the purification efficiency does not vary with the antigen because it is not the target of the purification. Rather, a quick and efficient purification can be easily performed focused only on HSP70.

An immunization strategy based on pHSP70-polypeptide complexes could be particularly useful in simply making universal vaccines against complex pathogens for which the protective

antigens are either difficult to make or not known. Even if the complexity of the preparation in terms of peptide composition (a technically unsolvable problem at present) could represent a major limitation to the possible use of this immunization strategy in humans, its application in the veterinary field could have interesting outcome.

Experimental procedures

Influenza NP cDNA cloning into the binary vector pGR106

The cDNA sequence encoding the NP of influenza A virus strain A/PR/8/34/Mount Sinai (H1N1) (GenBank accession no. AF389119) was excised from pBMSA-NP vector (Nakamura *et al.*, 1991) and inserted into pBluescript-SK+ plasmid generating pBS-NPREV. After propagation in *Escherichia coli* XL1 blue cells (Stratagene, Cedar Creek, TX), the NP-encoding sequence was excised from pBS-NPREV and transferred into the binary vector pGR106 (Lu *et al.*, 2003) generating pGR106-NP. The binary vector pGR106-NP was finally propagated in *A. tumefaciens* GV3101 (pSOUP) cells.

Nicotiana benthamiana plants agroinfiltration

NP protein was transiently expressed in plant tissues by agroinfiltration (Sparkes *et al.*, 2006). Briefly, exponentially growing *A. tumefaciens* GV3101 (pSOUP) cells carrying pGR106-NP were centrifuged (5000 *g* for 15 min) and pellet resuspended in infiltration buffer (10 mM 2-(*N*-morpholino) ethanesulphonic acid, 10 mM MgCl₂, pH 5.5). Groups of *N. benthamiana* plants at 40 days from germination grown in a containment P2 greenhouse under standard conditions (16 h light/8 h dark, 23 °C) were submerged in a beaker containing infiltration buffer alone or the bacterial suspension and subjected to 10 mmHg vacuum for 1 min in a vacuum bell jar (Sigma Chemical Co., St. Louis, MO) to induce bacteria penetration into the mesophyll forcing air out of the stomata. In the attempt to enhance transient expression efficiency, a group of plants was co-infiltrated with *A. tumefaciens* LBA4404 cells carrying a binary vector (p35S:p19) encoding the silencing suppressor protein P19 of TBSV (P19-TBSV) (Voinnet *et al.*, 2003). Leaves were collected at different time intervals, immediately frozen in liquid nitrogen and stored at –80 °C.

HSP70 and NP quantification in leaf extracts

Soluble protein extracts were prepared by grinding 200 mg leaf tissue in cold phosphate-buffered saline (PBS; 10 mM Na₂HPO₄, 10 mM NaH₂PO₄, 150 mM NaCl, pH 7.2) supplemented with 0.5 mM phenylmethanesulphonylfluoride (Fluka, St. Louis, MO) (Buffer A) as described previously (Buriani *et al.*, 2011). After TSP quantification using a Bradford colorimetric assay (BioRad, Hercules, CA), ELISA Maxisorp plates (NUNC, Roskilde, Denmark) were coated in triplicate with 50 µg TSP/100 µL per well of each sample. Positive control wells were coated with 200 ng human recombinant HSP70/100 µL per well (Sigma) or 200 ng recombinant NP/100 µL per well (Imgenex Corp., San Diego, CA). After washing and blocking, the plates were incubated with a monoclonal anti-HSP70 antibody (1 : 800) (clone BRM-22; Sigma) or with a monoclonal anti-NP antibody (1 : 200) (Imgenex) (or, as control, with an isotype-matched antibody of irrelevant specificity). Wells were then additionally incubated with horse radish peroxidase

(HRP)-conjugated anti-mouse IgG antibody (1 : 2500) (GE Healthcare Europe GmbH, Munich, Germany). Bound antibodies were revealed using 2,2'-azino-di-3-ethylbenz-thiazoline sulphate (ABTS; KPL Inc., Gaithersburg, MD), and the colorimetric reaction measured with an ELISA reader at 405 nm. Wells were considered positive when optical density values were at least three times above the background (PBS-coated wells).

Extraction and purification of HSP70 from plant tissues and peptide unloading

HSP70 were extracted from leaves through ADP chromatography in order to prevent the release of the naturally chaperoned peptides as previously described (Buriani *et al.*, 2011). Briefly, frozen plant leaf tissues were homogenized in cold PBS supplemented with 0.5 mM PMSF (Buffer A). The extracts were passed through Miracloth paper (Calbiochem, Darmstadt, Germany) and centrifuged at 4 °C, 20 000 *g* for 30 min, two times, to eliminate tissue debris. The supernatant was then passed through a G-25 column (GE Healthcare Europe GmbH) to change Buffer A in Buffer B (20 mM Tris-acetate pH 7.5 containing 20 mM NaCl, 15 mM β-mercaptoethanol and 3 mM MgCl₂), before loading on an ADP-agarose (Sigma Chemical Co.) column preswollen in Buffer B. After plant extract passage, the column was washed with 10 column volumes Buffer B, 10 column volumes Buffer B supplemented with 0.5 M NaCl, 10 column volumes Buffer B and then incubated 30' at room temperature with 1/3 column volume Buffer B supplemented with 3 mM ADP. At the end of the incubation, the elution was performed with five column volumes of Buffer B supplemented with 3 mM ADP. The collected fractions containing the protein (as verified by 12.5% (w/v) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining) were pooled, changed to PBS and concentrated using 50 000 molecular weight cut-off (MWCO) polyethersulfone (PES) concentrators (Vivaspin; Sartorius AG, Goettingen, Germany). HSP70 concentration was determined by the Bradford colorimetric assay (BioRad) and confirmed by SDS-PAGE and Coomassie blue staining.

Protein identity was verified by Western blot using the monoclonal anti-HSP70 antibody (Sigma) for detection. The absence of NP in the preparation was verified by ELISA performed as described in the section 'HSP70 and NP quantification in leaf extracts'. When necessary, peptide unloading from the SBD of the purified pHSP70 was performed through the addition of 3 mM ATP (Sigma), incubation for 1 h at 37 °C and peptides removal by passage through 10 000 MWCO PES concentrators (Sartorius AG) (Buriani *et al.*, 2011).

Endotoxin removal and evaluation of endotoxin contamination

Endotoxin was removed by using Endo Trap Red columns following manufacturer's instructions (Hyglos GmbH, Regensburg, Germany). Protein concentration in the finally collected flow through, determined by a Bradford colorimetric assay, was reduced of about 10%.

Plant HSP70 preparations, before and after endotoxin removal, were examined for endotoxin contamination using the classical LAL-based gel clot assay following manufacturer's instructions (Cape Cod Inc., East Falmouth, MA).

Immunization experiments

Six-week-old C57BL/6J and Balb/c female mice (Harlan, Udine, Italy) (3–6/group) were maintained under standard housing conditions in the ENEA Animal Care Unit. Mice were immunized subcutaneously (s.c.) into the tail base with 50 µL apyrogen saline containing different preparations of pHSP70 without adjuvant. To have an internal technical control in ELISPOT assay in each experiment, a group of mice was immunized with 50 µL apyrogen saline containing 50 µg of synthetic peptides ASNENMETM (C57BL/6J mice) or TYQRTRALV (Balb/c mice) (>95% purity) (ScyTek, Logan, UT) emulsified into IFA (Sigma).

Blood samples were collected by tail bleeds before immunizations and before sacrifice.

All experimental procedures repeated a minimum of three times were conducted in accordance with protocols approved by the ENEA ethical committee and by the Italian *Ministero del Lavoro, della Salute e delle Politiche Sociali*.

ELISPOT assay

A mouse IFN-γ ELISPOT kit was used according to manufacturer's instructions (eBioscience, San Diego, CA) as previously described (Lico et al., 2009). Briefly, after red blood cells lysis, 0.25×10^6 splenocytes were distributed in triplicate wells coated with an anti-mouse IFN-γ antibody and left unstimulated (negative control) or stimulated with synthetic peptides (0.4 µg per well) (ScyTek). After 20-h incubation at 37 °C, 5% CO₂ and 95% relative humidity, plates were washed and incubated with a biotinylated anti-mouse IFN-γ antibody followed by avidin-HRP. Spots were counted using an automated ELISPOT reader (Aelvis, Hannover, Germany). The frequency of peptide-specific T cells present in the responding population was calculated by subtracting the mean number of SFC counted in unstimulated wells from the mean number of SFC counted in stimulated wells and reported as number of specific SFC/10⁶ splenocytes. Cells were considered responsive only when the number of SFC in the wells stimulated with the peptide was at least two times that counted in the unstimulated control. Statistical analysis was performed using the unpaired Student's *t*-test. Differences were considered statistically significant when $P < 0.05$.

Antibody titration in sera

Overall, to determine anti-NP, anti-pHSP70 or anti-plant proteins IgG titres in serum samples, ELISA was performed as described above. Briefly, wells were coated with 50 µL PBS containing 1 µg of recombinant NP (Imgenex), pHSP70 (tested NP-free by ELISA performed as described in the section 'HSP70 and NP quantification in leaf extracts') or 50 µg of soluble plant proteins (depleted of pHSP70 through two times passage on an ADP-agarose column and verified to be pHSP70-free by ELISA performed as described in the section 'HSP70 and NP quantification in leaf extracts'). After washing and blocking, 50 µL of 1 : 10–1 : 400 dilutions of serum samples was added in triplicate to wells. Bound murine IgG were revealed by adding an HRP-labelled anti-mouse IgG polyclonal antibody (1 : 2500) (GE Healthcare) followed by ABTS (KPL). The colorimetric reaction was measured at 405 nm.

Endpoint titres were defined as the reciprocal of the highest serum dilution giving an absorbance three standard deviation above the blank (pre-immune sera). Mean titres ± standard

deviation were determined for each group. Differences among mean titres were evaluated through the unpaired Student's *t*-test and considered statistically significant when $P < 0.05$.

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